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Pro-aromatic Natural Terpenes as Unusual "Slingshot" Antioxidants with Promising Ferroptosis Inhibition Activity

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Ferroptosis is a cell death mechanism based on extensive cellular membrane peroxidation, implicated in neurodegenerative and other diseases. The essential oil component γ-terpinene, a natural monoterpene with a unique highly oxidizable pro-aromatic 1,4-cyclohexadiene skeleton, inhibits peroxidation of polyunsaturated lipid in model heterogeneous systems (micelles and liposomes). Upon H-atom abstraction, an unstable γ-terpinene-derived peroxyl radical is formed, that aromatizes to *p*-cymene generating HOO• radicals. As HOO• are small and hydrophilic radicals, they quickly diffuse outside the lipid core, blocking the radical chain propagation of polyunsaturated lipids. This unprecedented antioxidant "slingshot" mechanism explains why γ-terpinene shows a protective activity against ferroptosis, being effective at submicromolar concentrations in human neuroblastoma (SH-SY5Y) cells.

Ferroptosis is a cell death mechanism implicated in severe diseases such as neurodegeneration and ischemia-reperfusion injury.[1] The molecular basis of ferroptosis consists of the impairment of lipid hydroperoxides detoxification by glutathione peroxidase-4 (GPX4), a selenoprotein that catalyses the reduction of lipid hydroperoxides to their corresponding alcohols by glutathione. The simultaneous presence of hydroperoxides and labile iron causes an uncontrolled generation of free radicals that initiates the peroxidation of the phospholipid bilayer, ultimately leading to the loss of membrane integrity and cell death.[1] Chemical strategies to promote or suppress ferroptosis are receiving enormous attention, respectively, for enhancing the efficacy of cancer therapy, or to tackle neurodegenerative diseases.[1b] Many potent inhibitors of ferroptosis, like liproxstatin-1 (Lip-1), ferrostatin-1 (Fer-1), CuATSM, phenothiazines and phenoxazines, are effective radical trapping antioxidants (RTA) being able to suppress the peroxidation radical chain that is responsible for the membrane impairment.[2,3] From a mechanistic point of view, the RTAs investigated so far act by quenching the chain-propagating alkylperoxyl radical (LOO•) by either H-atom donation (Equation (1))[2b] or radical addition (Equation (2))[3] forming stabilized radicals unable to further propagate the oxidative chain. In the membrane interface, they can be regenerated by reducing species (X–H) such as $O_2^{\bullet-}$/HOO• or ascorbate (Equation (3)) thus explaining the superior activity of aromatic amines.[2b,3]

$$LOO^{\bullet} + RTA{-}H \longrightarrow LOOH + RTA^{\bullet} \quad (1)$$

$$LOO^{\bullet} + RTA \longrightarrow LOO{-}RTA^{\bullet} \quad (2)$$

$$RTA^{\bullet} + X{-}H \longrightarrow RTA{-}H + X^{\bullet} \quad (3)$$

Given the great interest toward ferroptosis inhibition, we wondered if this goal could be achieved by a completely different strategy, consisting of converting the LOO• radicals, that are confined in the membrane interior,[4] into hydrophilic hydroperoxyl (HOO•) radicals (see Scheme 1), which can also move to the water phase,[5] thereby interrupting the radical chain inside the lipidic particle ("slingshot" mechanism).

We tested this hypothesis by using γ-terpinene (γ-T, Scheme 1), a lipophilic monoterpene, found in the volatile components of plants used in human diet.[6] If compared to other hydrocarbons, γ-T is highly reactive as H-atom donor ($k_H \approx 1600\ M^{-1}\ s^{-1}$ at 30 °C)[7] and it forms, upon reaction with O_2, an alkylperoxyl radical (γ-TOO·) that breaks down, by a 1,4-intramolecular H-Atom Transfer (HAT), to HOO• and *para*-cymene (Cy) ($k_{1,4\text{-HAT}} = 4\times10^4\ s^{-1}$),[6,7] see Scheme 1. Importantly, Cy is another essential oil component commonly present in aromatic plants.[6] It is known that γ-T exhibits an antioxidant activity at relatively large (millimolar) amounts in homogeneous systems due to the formation of the two-faced oxidizing and reducing HOO• radicals. This activity can be attributed to

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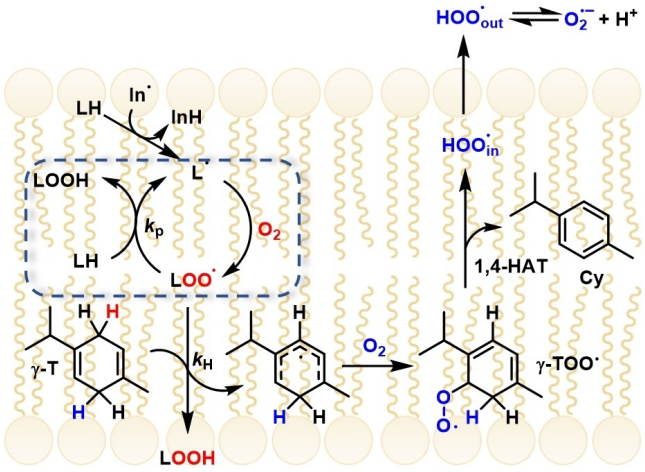

Scheme 1. "Slingshot" mechanism explaining the removal of lipophilic peroxyl radicals (LOO•) from the membrane by γ-terpinene (γ-T).

multiple mechanisms, such as the regeneration of antioxidants[8] or the acceleration of alkylperoxyl radical decay.[6] However, because HOO• can also propagate the oxidative chain, γ-T is a relatively weak antioxidant when used alone in homogeneous phases.[6] Here, we demonstrate that the "slingshot" mechanism causes γ-T to be far more active as an antioxidant in micelles and membranes than predicted from earlier studies, which accounts for its ability to protect against ferroptosis.

Firstly, we explored γ-T antioxidant effect in the autoxidation of methyl linoleate (MeLH) in Triton X-100 micelles at two different pH values, 7.4 and 4.5, representative of the pH of cytoplasm and of lysosomes, respectively, whose membrane damage is correlated to ferroptosis.[1e] The generation of radicals was provided by the water soluble initiator 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH). The peroxidation rate was followed by measuring by UV-vis spectroscopy the disappearance of the oxidation probe STY-BODIPY (Figure 1A–C).[2b] The addition of γ-T caused a approximately tenfold reduction of the probe consumption rate at both pH values, following an oxidation rate *vs.* concentration trend that could be explained in terms of co-oxidation kinetics (dashed lines in Figure 1B–C, see Table S1 for kinetic details).[9] The comparison of the antioxidant activities of γ-T at pH 4.5 and 7.4 demonstrates that although it is known that HOO• is a more active H-atom abstracting radical than $O_2^{\bullet-}$,[5a,d] the deprotonation of HOO• (pK_a = 4.69)[5a] is not essential for the "slingshot" mechanism. Reasonably, HOO• has limited ability to propagate the radical chain within the lipophilic micelle core, and it decays in the water phase. The inability of $O_2^{\bullet-}$ to propagate the oxidative chain was also verified by studying the peroxidation of the γ-T analogue 1,4-cyclohexadiene (1,4-CHD) in acetonitrile in the presence of bases of variable strength. The reaction was initiated by the decomposition of azobis(isobutyronitrile) (AIBN) and its rate was determined by measuring the O_2 uptake (Figure 2A, B).[3] The results showed that the O_2 consumption rate was reduced proportionally to the base strength (see Table S2) in agreement with the mechanism reported in Figure 2C. As expected, 1,4-CHD showed also an antioxidant effect against the peroxidation of MeLH in Triton X-100 micelles, although smaller than γ-T (see Figure S1).

Prompted by the good results obtained in micelles, the effect of γ-T on the peroxidation of phosphatidylcholine liposomes (PCL) from egg yolk, as a model of cell membranes, was also investigated. Egg-yolk PCL contained approximately 15% of polyunsaturated lipids,[10] and had a hydrodynamic diameter of 147 nm (Figure S2). The reaction was initiated by

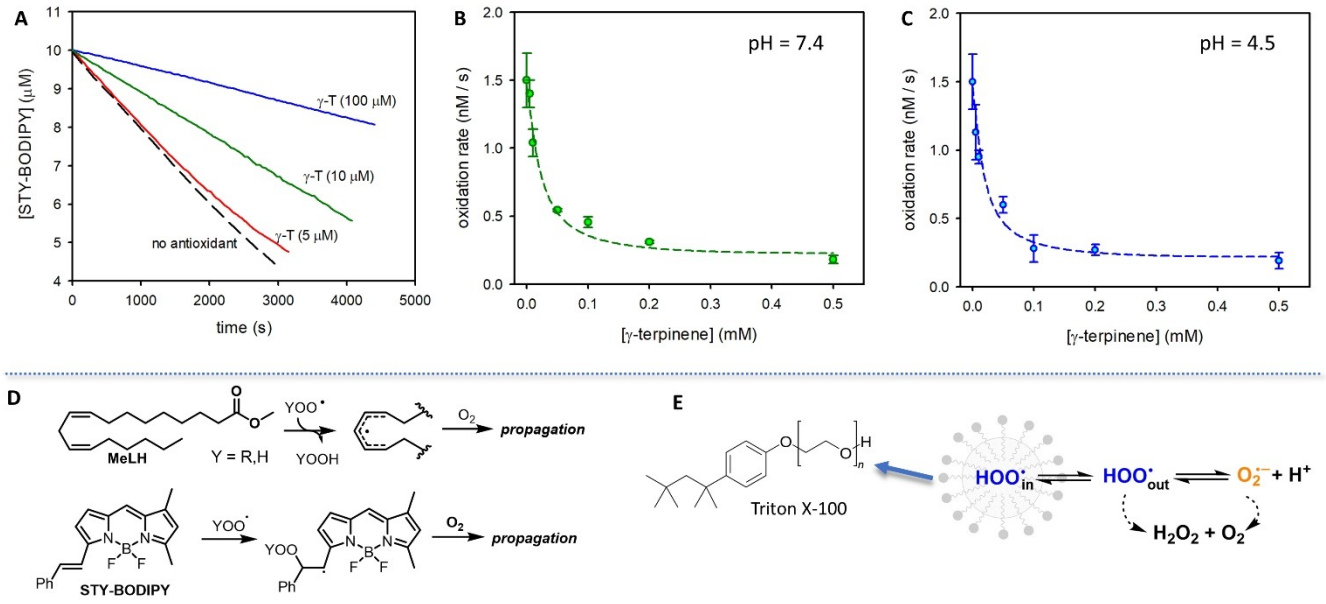

Figure 1. A. Consumption of STY-BODIPY (10 μM) during the autoxidation of MeLH (2.7 mM) in Triton X-100 micelles (8.0 mM) initiated by AAPH (10 mM) at 37 °C, in buffered water at pH 7.4. **B-C**. Rate of STY-BODIPY consumption as function of the concentration of γ-T, dashed lines represent the trend expected from a co-oxidation kinetic model. **D**. Oxidation mechanism of MeLH and STY-BODIPY. **E**. Effect of pH on HOO• exportation from the micelle to the water

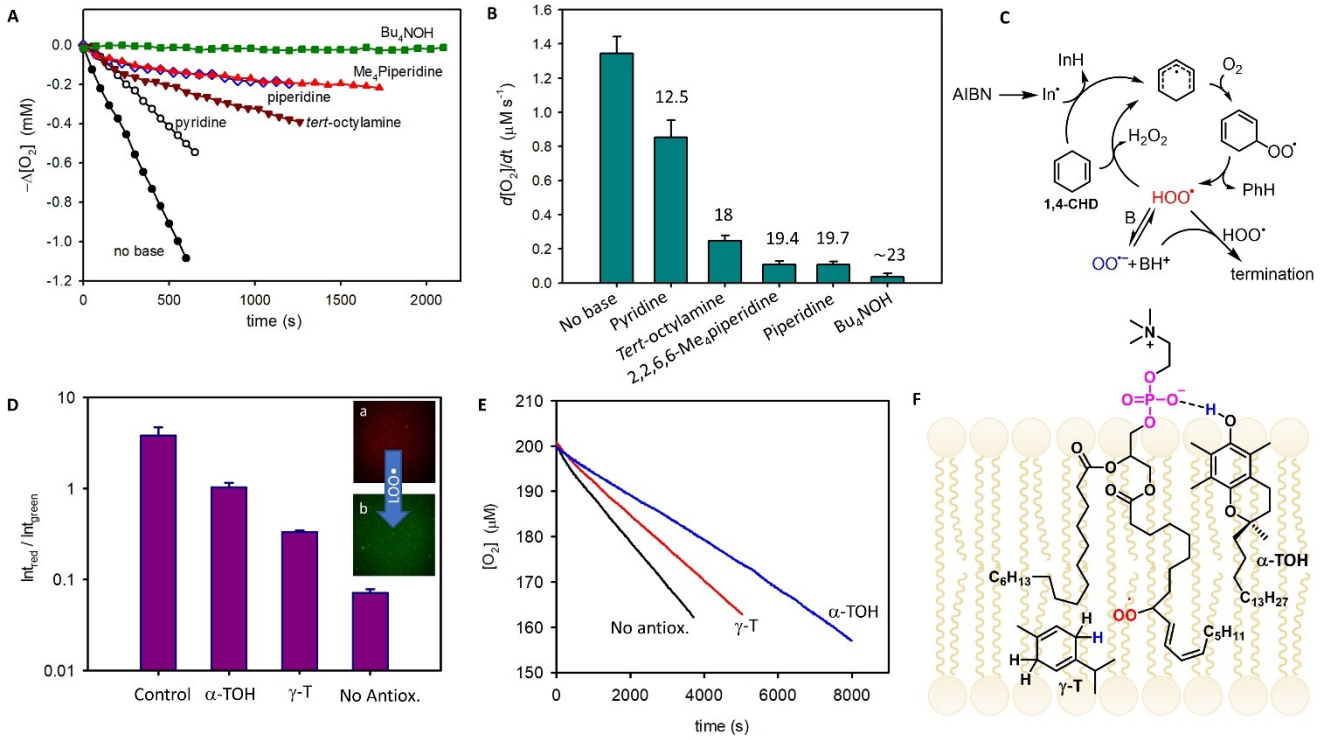

Figure 2. A, B. Oxygen consumption (**A**) and rates of O_2 consumption (**B**) recorded during the autoxidation of 1,4-CHD (0.26 M) in MeCN initiated by AIBN (25 mM) at 30 °C in the presence of bases (0.1 mM), whose protonated form has the pK_a reported in graph **B**. **C**. Mechanism of the effect of bases (B) on 1,4-CHD autoxidation, where In• is the initiating radical formed by decomposition of AIBN. **D, E**. Ratio between red (reduced) and green (oxidized) emission intensities of STY-BODIPY (1 μM) (**D**) and O_2 consumption (**E**) measured during the autoxidation of PCL (10 mM) initiated by AAPH (3 mM) at 37 °C and pH 7.4 in the presence of the antioxidants (5 μM). **F**. Different localization in PCL bilayer of α-TOH and γ-T.

AAPH and was monitored either by studying by a confocal microscope the fluorescence emission change of STY-BODIPY from red to green upon oxidation,[2b] and by measuring dissolved O_2 by a miniature fluorescence oxygen sensor.[11] Both experiments indicated that γ-T had an antioxidant effect slightly lower than α-tocopherol (α-TOH), one of the most important physiological antioxidant (Figure 2D, E). From the kinetic analysis of O_2 consumption plots (Figure 2E and S3), the inhibition rate constant (*i.e.* the reaction with LOO•) of γ-T was calculated as $(2.4 \pm 0.4) \times 10^3$ M^{-1} s^{-1} and that of α-TOH as $(4.5 \pm 0.6) \times 10^3$ M^{-1} s^{-1}, the latter being in good agreement with literature data[2c] (see Table S3 for kinetic details).

Interestingly, in chlorobenzene, an apolar organic solvents, α-TOH has a rate constant of reaction with LOO• of 3.2×10^6 M^{-1} s^{-1},[12a] i.e. about 1000 times larger than that of γ-T in the same solvent. However, previous studies showed that α-TOH, being able to form H-bonds with the phosphate groups in PCL,[2c] is subject to a strong kinetic solvent effect (KSE) that dramatically reduces its reactivity (Figure 2F).[2c] On the contrary, our results show that there is no KSE for the reaction of γ-T with LOO•, because the reactive moieties are C–H groups. Moreover, thanks to its hydrocarbon structure, γ-T resides in the lipophilic portion of the membrane, where LOO• are mainly found.

The antioxidant effect of γ-T experimentally observed has two important mechanistic implications: i) the lifetime of the peroxyl radical formed from γ-T (γ-TOO• in Scheme 1) is too short to allow γ-TOO• to propagate the oxidative chain; ii) the HOO• radical can escape from the membrane before reacting with polyunsaturated fatty acids. Assuming that γ-TOO• and HOO• can abstract H-atoms from linoleate with $k \approx 62$ M^{-1} s^{-1},[12b] and that the bisallylic group concentration in PCL is ~0.54 M, their half-life due to (hypothetical) chain propagation would be $(62\ M^{-1}\ s^{-1} \times 0.54\ M)^{-1} = 3 \times 10^{-2}$ s.[13a] In the case of γ-TOO• the half-life due to its fragmentation is $(4 \times 10^4\ s^{-1})^{-1} = 2.5 \times 10^{-5}$ s, therefore this process occurs faster than H-atom abstraction from linoleate. Regarding the HOO• radical, the time required for its diffusion outside the bilayer (t_D), can be estimated to be as low as 5×10^{-8} s by Equation (4),[13b] where D is the diffusion coefficient of HOO• ($D = 2 \times 10^{-5}$ cm^2 s^{-1})[13b] assumed equal to that of HO•, and the diffusion distance (d) is half the thickness of the bilayer (~22 nm).[13c]

$$d = 2.26(D\ t_D)^{1/2} \quad (4)$$

These considerations support the view that the escape of HOO• from the bilayer is a much faster process than any hypothetical propagation, and fully justify the "slingshot" mechanism depicted in Scheme 1.

The protecting effect of γ-T on PCL peroxidation led us to investigate its antiferroptotic effect in view of its possible use as neuroprotective agent. We pretreated neuroblastoma cells (SH-SY5Y), an in vitro model used in the study of mechanisms underlying ferroptosis in neurological diseases,[14] with different

concentrations of γ-T and induced ferroptosis using RSL3, a GPX4 inhibitor.[15] The data reported in Figure 3A show that γ-T is effective in counteracting SH-SY5Y cell death at a concentration as low as 50 nM. To further investigate the protective effect of γ-T on cell membranes, we studied the oxidation of the BODIPY–C11 probe,[16] that localizes in cell membranes where it can be oxidized by peroxyl radicals resulting in a shift of the fluorescence emission peak from ~590 nm (red) to ~510 nm (green). SH-SY5Y cells pretreated with vehicle and then treated with RSL3 (500 nM, 3 h) exhibited probe oxidation, while pre-treatment with 400 nM γ-T reduced probe oxidation (Figure 3B). Figure 3D shows representative images of SH-SY5Y cells stained with the BODIPY–C11 probe. It is evident that cells pre-treated with γ-T exhibited less oxidized probe (green) than controls after induction with RSL3. However, the antiferroptotic effect of γ-T declined rapidly over time, disappearing after 24 h from treatment (Figures 3 B,C), unlike Fer-1, that provided protection up to 24 h. This observation is consistent with the "slingshot" antioxidant mechanism, wherein γ-T functions as a sacrificial reductant that becomes inactive upon the formation of Cy. On the contrary, the aromatic amine Fer-1 can be regenerated by physiologic reductants as depicted in Equation (3).[2] The synthetic analogue 1,4-CHD also showed antiferroptotic activity and membrane protection (Figure S4), although with a lesser extent in comparison to γ-T, suggesting that the pro-aromatic moiety is at the origin of the biological effect. In order to elucidate the antioxidant mechanism of γ-T, which could be mediated not only by the "slingshot" effect, but also by the induction of enzymes involved in the cellular antioxidant response, we investigated whether treatment with γ-T could induce the expression of antioxidant enzymes involved in glutathione metabolism (glutathione peroxidase 4, GPX4 and glutathione reductase GSR) and Nrf2, a key transcription factor that controls the expression of genes whose

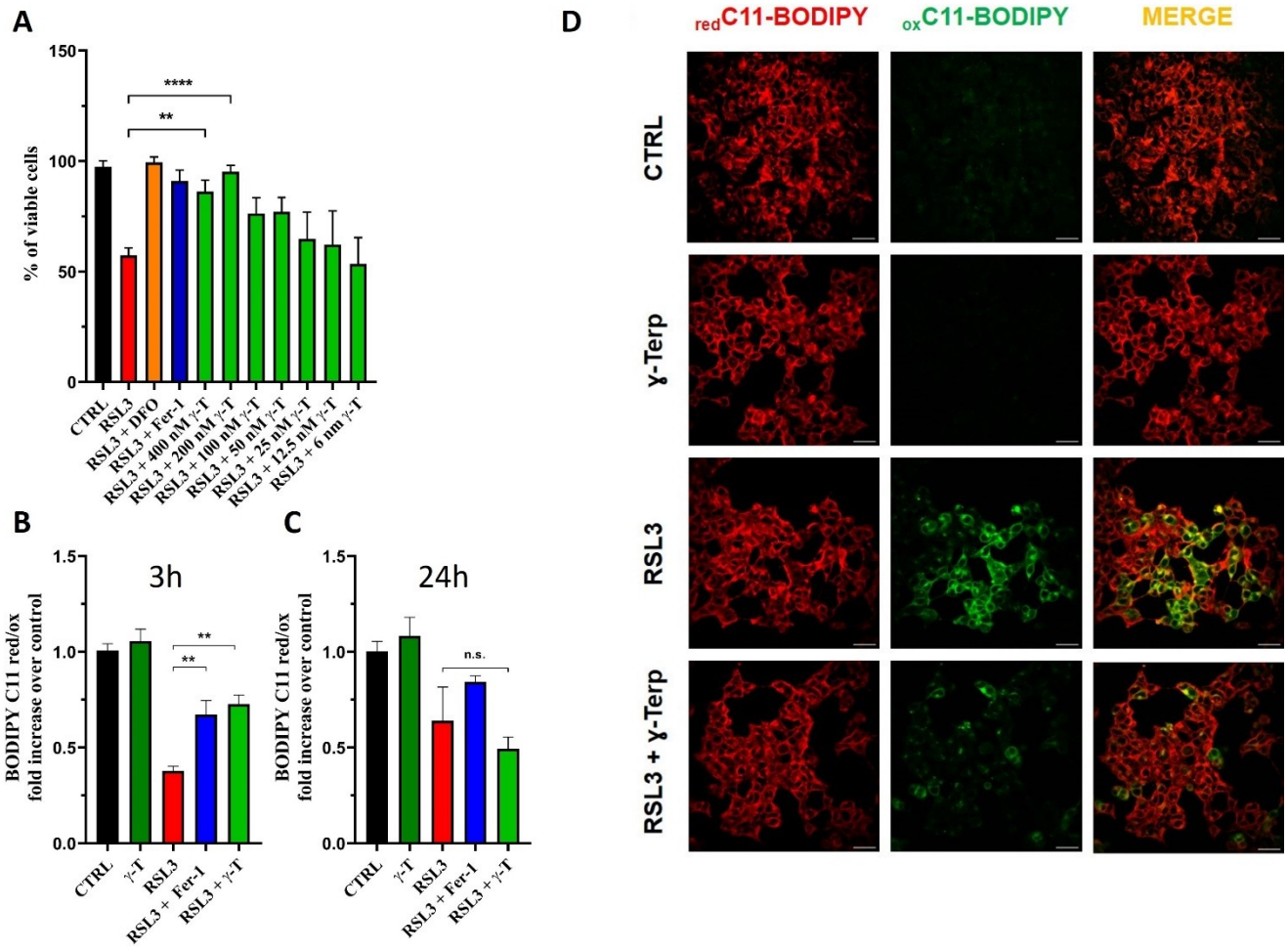

Figure 3. A. Cell viability determined by resazurin assay. SH-SY5Y cells were pre-treated with different concentrations of γ-T for 1 hour and then incubated for 18 hours with 400 nM RSL3. Pre-treatment for 1 hour with the iron chelator deferoxamine (DFO) (50 μM) or with Fer-1 (400 nM) was used as a positive control. Data are reported as mean ± s.e.m. (n = 6, one-way ANOVA ** $p \leq 0.01$; *** ≤ 0.001 vs. RSL3). **B-C**. Membrane peroxidation determination using BODIPY–C11. Cells were pre-treated with 400 nM γ-T or 400 nM Fer-1 for 1 hour and then incubated for 3 hours (**B**) or 24 hours (**C**) with 400 nM of RSL3. Data are reported as fluorescence intensity ratio of reduced/oxidized BODIPY–C11 emission (mean ± s.e.m., n = 4, one-way ANOVA, ** $p \leq 0.01$). **D)** Representative confocal images of live SH-SY5Y cells stained with BODIPY–C11. Cells were pre-treated with 50 nM γ-T and then incubated for 6 hours with 300 nM RSL3. Scale bars correspond to 30 μm.

protein products are involved in the detoxification and elimination of reactive oxidants. By performing reverse transcriptase - polymerase chain reaction (RT-PCR) experiments, we found that the treatment with γ-T and 1,4-CHD did not alter transcription levels of these proteins in SH-SY5Y cells, strongly suggesting a direct antioxidant mechanism (Figure S5). Additional investigations are needed to establish if these promising results can be generalized with other cellular lines.

In conclusion, we propose that the antiferroptotic action of γ-terpinene lies in the molecule's ability to remove the lipophilic peroxyl radical from the membrane compartment and convert it to the hydrophilic hydroxyperoxyl radical, which can move to the aqueous phase and be deprotonated to the less reactive superoxide anion species. This breaks the radical chain in the membrane and, from the cell viability data, we can speculate that the superoxide anion is efficiently detoxified by superoxide dismutase (mitochondrial and cytosolic SODs).

In this sense, γ-T would function as a "radical-trapping" inhibitor of ferroptosis, albeit through a peculiar mechanism, similarly to other active molecules recently discovered.[18] The mechanism depicted herein allows to rationalise previously reported results about the antioxidant activity of γ-T in red blood cells and in low-density lipoproteins.[19] Most importantly, the antiferroptotic activity of γ-T discovered herein calls for further studies of its neuroprotective activity. Although more research is needed to generalize our preliminary observations, especially regarding the short protection time provided by γ-T, the high lipophilicity and low molecular weight could provide high absorption and easy crossing of the blood brain barrier, that is a critical issue for drugs directed to the nervous central system.[20] In addition, these results might help to explain why certain essential oils containing γ-T as minor component, including bergamot and coriander ones, have neuroprotective activity even when simply inhaled,[21] and therefore could provide a rationalization for the pharmacological activity of extracts of aromatic plants of traditional medicine. Moreover, the reactivity principles enunciated herein are applicable to other easily oxidizable compounds, able to form $HOO^{\bullet}$ by 1,4-HAT, including reduced Coenzyme Q and vitamin K, which are object of intense research in the field of ferroptosis inhibition.[5,22]

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Conflict of Interests

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords: Superoxide · Ferroptosis · Lipid peroxidation · Antioxidant · Essential oils

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